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REVIEW

The Physiological Role of the Creatine Kinase System: Evolution of Views

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Abstract—The development of ideas concerning the buffer and transport functions of the creatine kinase system is described. The concept of ATP compartmentation at sites of its production and utilization is critically analyzed. Kinetic, thermodynamic, and structural data used as a basis for a hypothesis on the structural and functional coupling of mitochondrial creatine kinase and adenine nucleotide translocase are comprehensively analyzed, and experimental evidence inconsistent with this hypothesis is presented. It seems that the mitochondrial creatine kinase may serve to equilibrate ADP concentration in the intermembrane space with fluctuating ADP concentrations in the cytoplasm. It is suggested that creatine kinase molecules bound to other intracellular structures (e.g., to myofibrils) may equilibrate local ADP concentrations with those present in the cytoplasm.

Key words: creatine kinase, physiological role, adenine nucleotides, compartmentation, adenine nucleotide translocase, functional coupling, intracellular energy transport

The history of the study of creatine kinase (CK) is interesting and unusual. It started more than seventy years ago with the discovery of creatine phosphate (CP) [1, 2]. In 1934-1936, Lohmann and Lehmann described the creatine kinase reaction [3-6]. However, even now new functions of the creatine kinase system are being found, and therefore this story goes on.

The development of ideas on the physiological role of the creatine kinase system was always tightly bound to achievements in muscle physiology and bioenergetics. At the same time, studies of the creatine kinase system have made substantial contributions to ideas concerning the general arrangement of metabolic pathways and metabolic control in cells.

In this review, we chronologically summarize and critically analyze trends in modern concepts on the physiological role of the creatine kinase system. We also present our own viewpoints on some aspects of the functioning of the creatine kinase system.

Abbreviations: ANT) adenine nucleotide translocase; Arg-P) arginine phosphate; CK) creatine kinase (ATP–creatine phosphotransferase; EC 2.7.3.2); Cr) creatine; CP) creatine phosphate; mt-CK) mitochondrial creatine kinase.

BUFFER FUNCTION OF THE CREATINE KINASE SYSTEM

The history of concepts on the physiological role of the creatine kinase system can be subdivided into two periods. The first, "classic", period covers about forty years (from the late 1930s to the late 1970s). The major ideas of this period were based on studies of the skeletal muscle enzyme. Since more than 90% of CK activity in muscles is located in the cytoplasm, it was the story of the physiological role of cytoplasmic CK (see for review [7, 8]). The major conclusions of this period were as follows.

1. The creatine kinase system serves as a pH buffer [9].

2. The creatine kinase system also functions as an energy buffer [3, 10]. ATP is the direct energy source for muscle contraction [11]. CK constitutes about 10% of the total soluble cytoplasmic protein [12, 13], and its activity is much higher than ATP synthesizing and consuming processes [13]. So, in skeletal muscles the creatine kinase reaction is close to thermodynamic equilibrium. The thermodynamic equilibrium constant of the creatine kinase reaction is $2.81 \cdot 10^{-10}$ ($K = [H^+][MgADP^-]\,[CP^{2-}]/[MgATP^{2-}][Cr]$) [14]; under physiological conditions ($[Mg^{2+}] = 1$ mM, pH 7.0, temperature 38°C, and $I = 0.25$), the apparent equilibrium constant of this reaction ($K_{app} = [ADP][CP]/[ATP][Cr]$) is 0.006 [15].

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Thus, under physiological conditions the equilibrium of the creatine kinase reaction is shifted to ATP synthesis. At the same time, the concentration ratio of free ATP (5-10 mM) and ADP (37 μM) is greater than 100 [16]. The important consequence of these two facts is that during a transition from rest to muscular work a slight change in ADP concentration causes a significant change in the concentrations of CP and Cr, whereas the ATP concentration remains essentially unchanged until complete exhaustion of the CP stores. This is how CP acts as an ATP buffer.

The function of CK as an energy buffering mechanism means that, under metabolic conditions, CK maintains the ATP/ADP ratio (and consequently, the phosphoryl potential) at a high level [17].

TRANSPORT FUNCTION OF THE CREATINE KINASE SYSTEM

The modern period of studies on the creatine kinase system is characterized by revision of the "classic" notions. It started in the late 1960s when myocardium became a research object for physiologists.

In the late 1960s and in the 1970s it became clear that most (if not all) so-called "soluble" proteins interact with each other and with intracellular structures [18, 19]. It was suggested that a compartment is not necessarily separated by a membrane (e.g., as is the case for mitochondria); rather, a compartment can be a microenvironment within a region of tightly (or even poorly) interacting proteins and nearby intracellular surfaces. Such compartments have been called metabolic, functional, or micro-compartments [20]. The micro-compartment does not have clear physical boundaries, but it maintains complete or partial kinetic isolation [21].

Ideas concerning metabolic compartmentation have been widely used for analysis of the functioning of the creatine kinase system in myocardium.

In 1968, Gerken and Schlette [22] found that myocardial fatigue during the perfusion of rabbit heart with a solution containing a CK inhibitor occurs when ATP concentration is reduced by only 15-20%. They suggested that a major proportion of intracellular ATP is inaccessible for contractile proteins because adenine nucleotides do not freely diffuse from sites of their synthesis to places of their consumption.

In 1970, Gudbjarnason et al. [23] demonstrated that ischemic myocardium does not contract when stores of CP are exhausted and the ATP concentration drops to 80% of the control level. These data also seemed to support the idea of ATP compartmentation in myocardial cells.

Six years earlier, in 1964, CK isoenzymes—three cytoplasmic forms (MM, MB, and BB composed of two types of subunits [24]) and a mitochondrial isoenzyme (mt-CK) [25]—were recognized.

Subsequent studies on muscle and myocardial mitochondria revealed that Cr stimulates mitochondrial respiration due to involvement of mt-CK localized on the outer surface of the inner mitochondrial membrane [26, 27] in effective regeneration of ADP [26-28]. This suggested that, under physiological conditions, macroergic phosphate should leave mitochondria and be transported to "consumers" in the form of CP rather than ATP. It was also postulated that energy-consuming cell compartments should contain CK providing reversed phosphoryl transfer from CP to ADP. Indeed, experiments revealed that in skeletal muscles and myocardium CK is associated not only with mitochondria but also with myofibrils [29-31], sarcoplasmic reticulum [31-33], plasma membrane [34], and the nucleus [35]. In the heart, the cytoplasm contains half of the MM- and all of the MB- and BB-CK forms; their activity constitutes 40-50% of the total CK activity [30, 36]. Mitochondria contain 20-40% of the total CK activity [30, 36], and the rest is distributed among other intracellular structures [30, 36]. Skeletal muscle mitochondria contain only 2.5-6% of the total CK activity (reflecting lower mitochondria content in this tissue) [37, 38], but the specific activity of skeletal mt-CK is roughly the same as in the heart [37, 39]. In myocardial and skeletal muscle myofibrils of mammals, MM-CK is an integral component of M-line [40]; in skeletal muscles overall this CK constitutes only 5% of the total CK activity [40], whereas the major proportion of the activity is located in the cytoplasm.

Studies of the physiological potential of myofibril-associated CK (see for review [41]) revealed that ATP formed in the creatine kinase reaction has preferential access to the active sites of myosin ATPase (compared with exogenous ATP) [42, 43], whereas ADP formed in the ATPase reaction is more available to CK than to exogenously added pyruvate kinase [44]. In the presence of CP and ADP, myofibrils contract more rapidly and potently and relax better than in the presence of ATP alone (even taken at high concentrations) [43]. In the presence of CP and endogenous CK, the concentrations of ATP required for myofibril relaxation are lower than in the absence of CP [45, 46] or in the presence of added pyruvate kinase system [46]. In the presence of CP, the K_m value of myofibril ATPase for MgATP is reduced [47]. It was concluded that CK and the corresponding ATPases are incorporated into functional compartments that have limitations for adenine nucleotide diffusion. The concentrations of adenine nucleotides in these compartments, maintained by the catalytic activity of CK at levels distinct from those in cytoplasm, determine the functional activity of the ATPases, in particular of myosin ATPase [43, 44, 48].

These studies led to the idea of a transport function of the creatine kinase system (Fig. 1). According to this idea, adenine nucleotides are compartmentalized in places of their synthesis and consumption: under non-

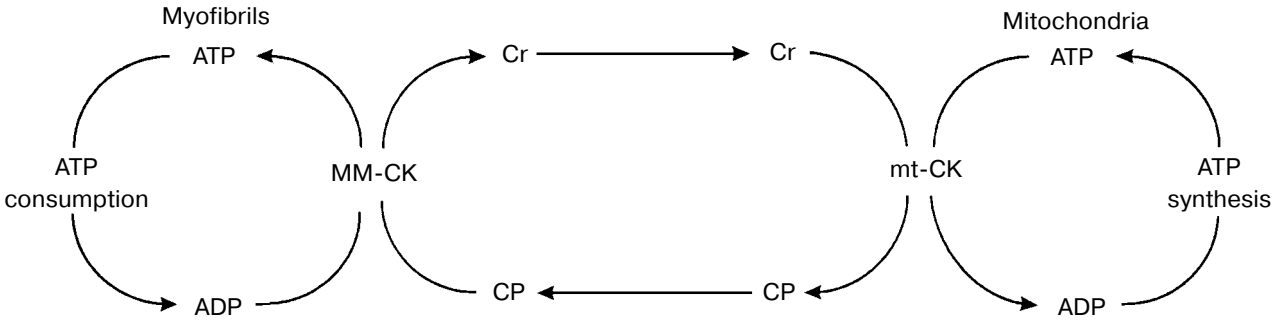

Fig. 1. Proposed transport function of the creatine kinase system. See explanation in text.

equilibrium conditions, MM-CK in myofibrils and mt-CK function in a unidirectional way (as indicated by the arrows in Fig. 1). Macroergic phosphate cycling between places of synthesis and consumption occurs via the "phosphocreatine shuttle" [49].

HYPOTHESIS ON THE UNITY OF BUFFER AND TRANSPORT FUNCTION OF CK

The transport function of the creatine kinase system, as it is shown in Fig. 1, excludes the buffer function. In fact, the buffer role of CP suggests free movement of all reactants of the creatine kinase reaction in the muscle cell cytoplasm and a reaction state close to equilibrium [50]. Attempts to solve this contradiction by NMR spectroscopy gave unequivocal results: earlier studies indicated the possibility of compartmentation [51], whereas later studies disproved it [50, 52, 53].

Using the equation of facilitated diffusion, Meyer et al. [54] demonstrated the possibility of facilitated diffusion of adenine nucleotides provided that they are in equilibrium with CP and Cr and diffuse gradients of ATP and ADP in the muscle fiber are related to spatial separation of ATP synthesizing and consuming processes. They showed that the relative contribution of guanidine compounds and adenine nucleotides to macroergic phosphate transfer between places of its synthesis and consumption is determined by the overall concentration of each couple of compounds, the apparent equilibrium constant of the creatine kinase reaction, and the ATP/ADP concentration ratio. Taking into consideration all these factors, CP should be the main form of macroergic phosphate transport from mitochondria to myofibrils under physiological conditions, whereas Cr is the main form diffusing in the reversed direction. Thus, according to the viewpoint of these authors, the buffer and transport functions of CP are interdependent, and the fact of the transport function of CP does not mean and does not require adenine nucleotide compartmentation. It was suggested that the functioning of the shuttle mechanism requires just the presence of CK in any cell compartment involved in ATP turnover, and special CK localization in places of ATP synthesis and consumption is not needed [17, 54]. Meyer et al. [54] suggested that CK binding at places of ATP production or consumption is needed for the increase in local enzyme activity at places where energy flux is maximal; this provides nearly equilibrium conditions of the reaction at lower total enzyme activity than would be in the case of equal distribution of CK within the cell.

These authors called the buffer function of the creatine kinase system the "spatial" buffer function because it buffers ATP and ADP concentrations in any region of the cell. The "spatial" buffer function means that in the presence of the active creatine kinase system, the maintenance of any given diffusive flux of macroergic phosphates requires smaller gradients of ATP and ADP concentrations than in the absence of CK [54]. However, the "classic" buffer function is a "temporal" function that does not consider diffusion gradients of ATP and ADP and operates only by buffering concentrations of ATP and ADP [54].

The importance of the "spatial" buffer function of CK has been analyzed by several groups [17, 54-57]. Calculations revealed that the transport function is ultimately important only in the case of rather long diffusion distances, as in fast twitch fibers, where the thickness of a myofibril bunch varies from 20 to 30 μm [54, 56], and in neuronal axons. In the case of slow twitch fibers of skeletal muscles and myocardium, where each myofibril is surrounded by mitochondria and its thickness is only 1-2 μm [54, 55], energy transport could occur via simple diffusion of ATP and ADP molecules [54, 56, 57], and therefore the transport function in the heart is not important [54]. Recently this conclusion was confirmed in direct experiments [58].

Saks [59] made a suggestion supported by others [60] that energy exchange between places of its accumulation and consumption occurs as a "metabolic wave", a series of cyclic changes in ADP and Cr concentrations which

are vectorially distributed from myofibrils to mitochondria and simultaneous corresponding changes in CP and ATP concentrations directed from mitochondria to myofibrils. These local changes occur as the result of phosphoryl group transfer sequentially produced by neighboring molecules of cytoplasmic CK catalyzing the reaction, which is close to equilibrium. This is a questionable suggestion. Indeed, in an open system (as each cell is), the equilibrium condition of the creatine kinase reaction means many turnovers of the CK reaction (both in the forward and reversed directions) per turnover of ATPase or ATP synthase. Equilibrium also means that the diffusion of substrates does not limit the reaction rate. Thus, the equilibrium creatine kinase reaction in the cytoplasm of muscle cells is a scalar process, but a scalar process cannot generate a vectorial movement of metabolites [61].

ANALYSIS OF THE HYPOTHESIS OF ATP COMPARTMENTATION

If the transport function of the creatine kinase system is determined by the equilibrium creatine kinase reaction, what should be done with the ATP compartmentation that has been demonstrated in many experiments? But another question arises—was ATP compartmentation actually demonstrated in those experiments? The experiments by Gerken and Schlette [22] could be explained by ATP compartmentation if we assume that about 80% of myocardial ATP is tightly bound and inaccessible for exchange and diffusion. However, this assumption conflicts with the following experimental data.

1. Arrio-Dupont and De Nay [62] found that, during fractionation of frog myocardiocytes, 90-92% of the ATP is detected in the cytoplasm and the rest in mitochondria regardless of the functional state of the cells. These results are consistent with ATP content (10-15% of total) in heart mitochondria of homeothermic animals [63, 64]. At the same time, Arrio-Dupont and De Nay [62] found that the cytoplasm contains only 2.5% of the ADP; this is also consistent with the earlier recognized fact [16, 65, 66] that a significant proportion of the ADP in skeletal muscle and heart is bound to intracellular structures.

2. The overall content of ATP in striated muscles and myocardium is 8-10 mmole per kg of wet weight [67, 68], whereas the ADP content is about 1 mmole per kg [67, 68].

3. Using ^{18}O-exchange, it was shown that almost all ATP gamma-phosphate in diaphragm muscle is metabolically active, without respective to the functional state of this muscle [67], whereas in the resting muscle only 25% of the ADP is metabolically active [69]. This means that even if in native myocardiocytes part of the cytoplasmic ATP is bound to soluble proteins, this binding is not tight.

4. Calculations show that the portion of cytoplasmic ATP bound to soluble proteins cannot be large. In fact, it is known that a kilogram of muscles contains 40-50 g of soluble protein [70, 71]. Suppose that the average mass of these proteins is 50 kD; in this case the total content of these proteins is 0.8-1.0 mmole/kg. Even if we assume that each of their subunit binds an ATP molecule, the concentration of free ATP would be reduced by only 10%.

5. Under physiological conditions, during a contraction–relaxation cycle, a portion of the ATP may be bound to myosin. However, the concentration of active sites of myosin in myocardium and skeletal muscles is 150 [72] and 240 μM [73], respectively. This can give the same quantity of bound ATP.

6. The determination of diffusion coefficient ratios of ATP and CP in skeletal muscle and aqueous solution revealed that they are the same and do not exhibit any abnormalities compared with the ratios found for other compounds [74, 75].

7. ^{31}P-NMR experiments with skeletal muscles and myocardium suggest the possible existence of an NMR-invisible mitochondrial pool of matrix-associated ATP [76]. In rat heart, Suzuki et al. [77] found a slowly exchangeable ATP pool that represented about 30% of the total ATP pool. They suggest that this pool includes mitochondrial ATP and possibly ATP bound to cytosolic proteins [77]. Weisman and Kushmerick suggested that the NMR-invisible pool of ATP does not exceed 5-10% of the total ATP content [50].

Thus, results from different laboratories (obtained using various techniques) suggest that at least 80-90% of cytoplasmic ATP exists in the free state.

If the major proportion of muscle cell ATP exists in the free state, the question arises, whether formation of metabolic compartments near myofibrils and in the intermembrane space of mitochondria (with local concentration of ATP significantly differing from its average concentration in cytoplasm) is really possible? A partial answer came from experiments on isolated heart mitochondria.

Gellerich et al. [78] studied oxidative phosphorylation in a medium containing phosphoenolpyruvate and 300-fold excess (with respect to oxidative phosphorylation) of pyruvate kinase activity. Under these conditions, mt-CK functioning is accompanied by the appearance of local concentrations of adenine nucleotides in the intermembrane space; the maximal value of the gradient of concentrations between the intermembrane space and the external medium was about 13 μM [78]. (In the intermembrane space, ADP concentration was higher, whereas ATP was lower than in the external medium.) The concentration of free ADP in the cytoplasm of muscle cells is about 30 μM [16], so the increase in ADP concentration by 13 μM gives more than 30% increase in free ADP, whereas the changes in ATP concentration are insignificantly small [78]. An almost identical value of ADP concentration gradient between the intermembrane space and the external medium (12-13 μM) was found in stud-

ies of the adenylate kinase reaction in liver and heart mitochondria [79, 80]. Experiments with mt-CK have recently been reproduced in skinned heart fibers [81].

As shown in Bessman's laboratory [82, 83], at ATP concentration in the medium of about 500 μM, its compartmentation at the active site of mt-CK did not exceed 10-12% during oxidative phosphorylation. Thus, at ATP concentrations of about 500 μM, diffusion limitations cannot lead to significant ATP compartmentation in the mitochondrial intermembrane space.

The above-mentioned consideration could be questioned because the *in vivo* gradient of adenine nucleotide concentrations between the cytoplasm and the intermembrane space might be higher than in the *in vitro* experiments. In fact, K_m^{ADP} for oxidative phosphorylation in isolated heart mitochondria is 20-30 μM [84], but in skinned heart fibers and in slow twitch skeletal muscle fibers this parameter is one order of magnitude higher (300-400 μM) [85]. This difference was explained by the existence of a special cytoplasmic protein reducing the outer membrane permeability for ADP that is lost during isolation of mitochondria [85, 86]. This phenomenon was also observed in skinned myocardial fibers from various animal species [60, 87]. Gellerich et al. [84] showed that the addition of 15% dextran to the incubation medium of rat heart mitochondria (this imitates cytoplasmic oncotic pressure) was accompanied by an increase in of K_m^{ADP} for oxidative phosphorylation from 16 to 50 μM. They suggest that the K_m^{ADP} value for oxidative phosphorylation in intact myocardiocytes is about 50 μM and that the values reported for skinned muscle fibers are overestimated due to the complexity of the model used [84]. Using perfused rat heart, From et al. [88] found that the K_m^{ADP} value for oxidative phosphorylation is 25 μM under conditions when NADH production is not the respiration-limiting factor. Experiments with skinned fast twitch skeletal muscle fibers revealed that the K_m^{ADP} value for oxidative phosphorylation was roughly the same as in the case of isolated mitochondria [60, 85]. Nevertheless, all recognized differences [60, 84, 85, 87] are still within the micromolar concentration range, and so it is still reasonable to suggest that under physiological conditions the concentration gradient between the intermembrane space and cytoplasm is important for ADP but not for ATP, CP, or Cr.

The mitochondrial intermembrane space compartment is maintained not only by the existence of unstirred layers and protein–protein interactions as in the case of metabolic compartments formed by cytoplasmic proteins (including those localized near myofibrils). Limited permeability of the outer mitochondrial membrane is also crucial for its maintenance. In this regard, it is relevant to suggest that a gradient of adenine nucleotide concentrations, which may be maintained by some hypothetical metabolic compartments in the cytoplasm, will also be within the micromolar concentration range. It was shown that in the presence of dextran the diffusion gradient of ADP between the compartment formed by hexokinase bound to the external surface of the outer mitochondrial membrane and the external medium is about 12 μM [89].

Thus, is there any explanation other than ATP compartmentation for experiments demonstrating the termination of myocardial contraction during CK inhibition or CP exhaustion, when the ATP content is reduced by only 10-20% [22, 23]? One possible reason for the development of muscle fatigue is the increase in ADP concentration. If the steady-state concentrations of free ATP and ADP are 10 mM and 30 μM, respectively, hydrolysis of 10% of the ATP (if the creatine kinase system is not operating) should result in 30-fold increase in ADP concentration, and ADP is an inhibitor of the myofibrillar ATPase. The role of MgADP in the development of diastolic dysfunction has recently been analyzed by Tian et al. [90]. The heart is known to be more sensitive to a decrease in ATP concentration than skeletal muscles. This fact correlates well with the K_i^{ADP} values for heart and skeletal muscle actomyosin, 10 and 170 μM, respectively [41]. Among other factors, local acidification and P_i accumulation may be also important [41].

As to experiments on isolated myofibrils and skinned muscle fibers [42-46], where the presence of an active creatine kinase system significantly increased contraction parameters, it should be noted that these models are characterized by large diffusion distances [54]. So, the results of these experiments may well be explained by acceleration of macroergic phosphate exchange produced by the creatine kinase system rather than adenine nucleotide compartmentation. This is consistent with a mechanism proposed by Meyer et al. [54]; in some cases [43, 45, 46] the reduction of steady-state ADP concentration and general increase in macroergic phosphate transport played a major role, whereas in other cases [42] CP molecules as the transport form of macroergic phosphate were important.

Supporters of the idea of ATP compartmentation often quote reports by Jones [91, 92] as experimental evidence confirming their viewpoint. However, in these studies the diffusion concentration gradient of ATP appearing in liver cells after total suppression of ATP concentration induced by special treatments (hypoxia, etc.) rather than ATP compartmentation was analyzed [91]. Jones suggests [92] that under normal aerobic conditions gradients of ATP concentrations cannot be very high due to the high intracellular concentration of this compound.

Considering the metabolic consequences of ATP compartmentation, it is tempting to draw an analogy with macroenergetics. In bioenergetics, ATP is a universal energy source; electric energy plays a similar role in economics. In countries with well-developed economics, separate sources of electric energy are jointed into a united energy system; this provides rational use of material resources and energy. In bioenergetics the notion on a

free state of cytoplasmic ATP and equal availability of the total ATP pool to ATP consuming systems located within this compartment seems to correspond to this organization principle. The principle of ATP compartmentation extrapolated to macroenergetics may be formulated as follows: each energy consumer must have its own power station. This principle may also mean that energy consumers can get energy on the grounds of competition.

Interestingly, muscles have clear signs of a "united single energetic system" in the form of the mitochondrial reticulum [93]. The latter was found in diaphragm and skeletal muscles [93, 94]. Muscle mitochondria having peripheral localization also interact with the mitochondrial reticulum through their branches [93, 94].

Heart mitochondria lack such branches and do not form a united reticulum. However, they do form numerous contacts so that each myocardiocyte has several clusters of contacting mitochondria [95]. Skulachev [96] suggested that the existence of mitochondrial reticulum and clusters promotes rapid energy transfer (in the form of electrochemical potential of H^+ ions) from the cell periphery enriched with oxygen and substrates to mitochondria of the central parts, where this energy is utilized for ATP synthesis. Such energy transfer would be important under conditions of starvation (when substrate deficit exists) or intensive muscular work requiring rapid involvement of all mitochondria in ATP synthesis.

ATPases are characterized by a low value of K_m^{MgATP} compared with cytoplasmic ATP concentration. So, it was concluded that high concentrations of ATP are not important for muscle functioning [59]. However, the saturation of active sites of the corresponding enzymes with ATP under physiological conditions may also mean that the energetics of normally functioning muscle is arranged in a way such that ATP, the energy source, is not the rate-limiting factor.

HYPOTHESIS OF STRUCTURAL–FUNCTIONAL COUPLING BETWEEN mt-CK AND ANT

The concept of a role of structure-bound CK in compartmentation of adenine nucleotides has received the most comprehensive development in proposed mechanisms of mt-CK functioning. Mitochondrial CK is located on the external surface of the inner mitochondrial membrane [26, 27]. It is bound to the membrane by electrostatic forces [97-99]. It was initially suggested that mt-CK interacts with ANT, with which it shares common substrates [100]. However, a large voluminous inhibitor of ANT, carboxyatractyloside, did not influence mt-CK binding with mitochondrial membranes [97, 99, 102]. Subsequently, it was clearly recognized that in mitochondria mt-CK is bound to phospholipids [97, 103-105] rather than to ANT; and a negatively charged phospholipid, cardiolipin, is its receptor in the mitochondrial membrane [102, 106, 107].

Saks and Jacobus [100, 108, 109] suggest that the high efficiency of mt-CK is due to functional coupling with oxidative phosphorylation. According to their conception, this functional coupling stems from a structural interaction between mt-CK and ANT that occurs during oxidative phosphorylation due to frequent interactions of their molecules by lateral diffusion in the membrane [110]. During the interaction of these proteins, ATP synthesized in mitochondria is transferred from ANT to the active site of mt-CK, whereas ADP is transferred in the reversed direction (from mt-CK to ANT); this results in saturation of the active sites of both proteins with their substrates [59, 110].

Over the last decade this idea was further developed in the laboratories of Wallimann [111, 112] and Brdiczka [113]; they considered the role of mt-CK at the contact sites of the inner and outer mitochondrial membranes. The former group suggested [111, 112] that at these contacts mt-CK, ANT, and outer membrane porin forms a dynamic nucleotide transporting and transphosphorylating multienzymatic channel that directly links the mitochondrial matrix with the cytoplasm (Fig. 2). This channel is formed during lateral diffusion of the mt-CK octamer and the ANT dimer along the surface of the inner membrane and stabilized by an interaction with the porin pore in the outer membrane [112]. The number of ANT dimers interacting with one mt-CK octamer might vary from four to eight [112]. Active sites of mt-CK located in the central channel of the octamer accept electroneutral Cr via the cation-selective pore, and matrix ATP is transported by ANT molecules. ADP formed in the CK-reaction is transported by ANT to the mitochondrial matrix, and CP diffuses to the cytoplasm via non-regulated pores beyond the contact sites. The concentrations of ATP, ADP, and CP in the channel differ from those in the cytoplasm. This allows mt-CK to synthesize CP even at high CP/Cr ratio in the cytoplasm [113, 114]. Variations of this scheme differing by mutual locations of the components and details of the mechanisms of their functioning can be found in recent reviews [115, 116].

These hypotheses are based on the following experimental data.

The Saks–Jacobus hypothesis is based on kinetic and thermodynamic data. Kinetic analysis revealed that the rate of CP synthesis by mitochondria during oxidative phosphorylation is higher than in the presence of the inhibitor of oxidative phosphorylation oligomycin and external ATP [100, 117]. It was concluded that mt-CK utilizes ATP synthesized during oxidative phosphorylation more rapidly than exogenously added ATP. The observed reduction of mt-CK K_m^{MgATP} during oxidative phosphorylation [100, 118] was explained by the existence of higher local concentration of ATP in the region of the active site. The effect of oxidative phosphorylation

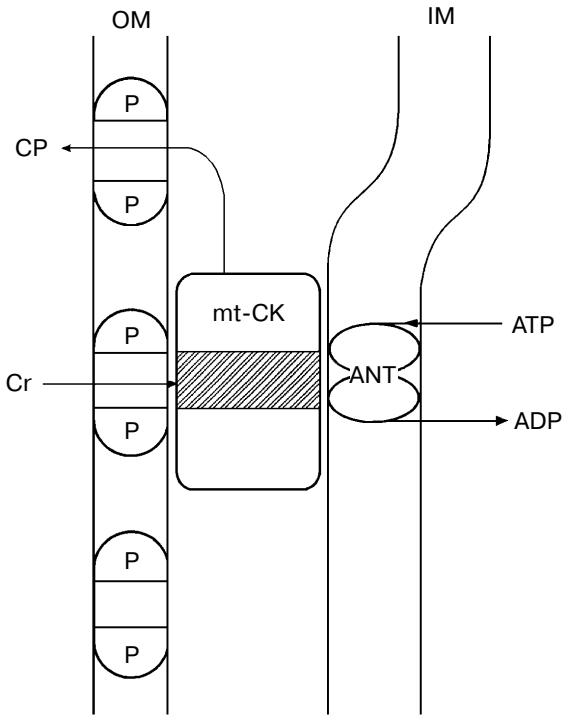

Fig. 2. Scheme illustrating asymmetrical functioning of a mitochondrial multienzyme channel formed by porin, mt-CK, and ANT. IM, OM, and P denote the inner mitochondrial membrane, outer mitochondrial membrane, and porin, respectively. Other explanations are given in the text.

on K_m^{MgATP} was abolished by 0.125 M KCl [119]. The latter phenomenon was explained as KCl-induced solubilization of mt-CK to the intermembrane space, which breaks the functional coupling [119].

Thermodynamic experiments revealed that during oxidative phosphorylation the acting mass ratio of reactants of the CK reaction measured in the incubation medium exceeds the K_{app} for the reaction of CP synthesis by 2.5-4.0-fold [120-122]. This effect was observed in experiments with mitochondria [120, 122] and mitoplasts [121, 122]. Inhibitors of the respiratory chain and oxidative phosphorylation, or anaerobic conditions abolished this disproportion [120-122]. It was concluded that during oxidative phosphorylation the real concentrations of ATP and ADP at the active site of mt-CK differ from those in the incubation medium (ATP concentration is higher whereas ADP concentration is lower at the active site of mt-CK compared to the medium). This allows mt-CK to synthesize an excess amount of CP and proves the existence of structural and functional coupling between mt-CK and ANT [120-122].

It was shown that antibodies against mt-CK inhibit ANT; this indicates the nearby location of the two proteins on the inner mitochondrial membrane [123].

The Wallimann–Brdiczka hypothesis is mainly based on structural data. It has been shown that the outer mitochondrial membrane is permeable for adenine nucleotides only at places where porin is localized; porin dimers form anion-selective pores (of 2-nm diameter) in the membrane [124-126]. Contact sites between the outer and inner mitochondrial membrane contain porin, mt-CK, and ANT [127, 128]. The number of contact sites depends on the energy state of mitochondria [125, 129]. The permeability of porin pores for anions in the contact sites depends on the inner membrane potential and is reduced during oxidative phosphorylation, when the pores become cation-selective [125, 130]. In contrast to cytoplasmic forms (which are dimers), mt-CK may exist as dimers and octamers [39, 111, 112, 131, 132]. The octamer is a perforated cube with a central cavity 2.0-2.5 nm in diameter [105, 112, 133]; the mt-CK octamer may interact with the outer and inner mitochondrial membranes [105, 134] and also with porin dimers [135].

ANALYSIS OF THE HYPOTHESIS OF STRUCTURAL–FUNCTIONAL COUPLING BETWEEN mt-CK AND ANT

Arguments supporting the existence of structural–functional coupling between mt-CK and ANT must be critically analyzed.

As a rule, the results of kinetic experiments have strongly depended on experimental conditions, and thus their interpretation is difficult. For example, Altshuld and Brierley [136, 137] and also Borrebaek [138, 139] have demonstrated that the reduction of the rate of CP synthesis in the presence of oligomycin observed by Saks et al. [100, 117] is attributed to the increase in ADP concentration in the incubation medium. Addition of a large excess of pyruvate kinase in the presence of phosphoenolpyruvate abolished the inhibitory effect of oligomycin [136-139]. Extrapolation to arbitrarily high concentration of pyruvate kinase gives a rate of CP synthesis exceeding the same parameter determined for phosphorylating mitochondria [137]. Lipskaya et al. [83], using pH-metric registration of the initial rate of CP synthesis, also found that in oligomycin-treated mitochondria it exceeded the rate of CP synthesis in phosphorylating mitochondria [83]. Experiments on transgenic mice lacking MM-CK revealed that the flux though mt-CK significantly exceeded the maximal rate of mitochondrial respiration (this was shown using ^{31}P-NMR spectroscopy) [140].

The decrease in K_m^{MgATP} observed during oxidative phosphorylation can be explained in various ways. For example, it may be attributed to oxidative phosphorylation-induced partial release of membrane mt-CK to the intermembrane space; another possibility is the dissociation of octamers to dimers because these transitions are accompanied by similar changes in kinetic parameters of

the enzyme [103, 141]. The reduction of negative surface charge on the inner mitochondrial membrane observed during oxidative phosphorylation may also contribute to the decrease in K_m for the negatively charged MgATP molecule [142].

Potassium chloride is not a specific agent for solubilizing mt-CK. Many other substances can solubilize this enzyme [97-99, 143], and this effect depends on ionic strength rather than on the chemical nature of the substances [97-99]. If functional coupling between mt-CK and ANT disappears in the presence of KCl, this questions the possibility existence of such coupling *in vivo* under conditions of physiological ionic strength. Unfortunately, the choice of KCl as the solubilizing agent was unlucky because Cl^- is a competitive inhibitor with respect to MgATP for both cytoplasmic [144] and mt-CK [145]. So the increase in K_m^{MgATP} in the presence of KCl [119] may be related to the inhibitory effect of Cl^-.

A more definite answer to the question of the existence of structural–functional coupling between mt-CK and ANT comes from radioisotope studies of ATP compartmentation in the active site region of mt-CK during oxidative phosphorylation [82, 83, 136, 146]. In these experiments, mitochondria were incubated for a short time in medium promoting oxidative phosphorylation and the CK reaction in the presence of unlabeled ATP and labeled P_i. Oxidative phosphorylation was accompanied by the formation of labeled ATP. Comparison of label incorporation to external medium ATP and newly synthesized CP allowed the contribution of ATP formed during oxidation phosphorylation to CP synthesis to be evaluated; this allowed the extent of ATP compartmentation in the active site region of mt-CK to be evaluated [82].

If adenine nucleotide exchange between active sites of mt-CK and ANT is related to their direct transfer within an mt-CK–ANT complex, the extent of ATP compartmentation in the active site of mt-CK must not depend on the concentration of external ATP and must be 100%. At least it should increase with the increase in oxidative phosphorylation rate that occurs, for example, during an increase in ATP concentration in incubation medium containing Cr. However, isotope experiments by Bessman's group [82] revealed that the increase in ATP concentration has the opposite effect on the extent of compartmentation, which is reduced. The maximal extent of compartmentation (during extrapolation of ATP concentration to zero) was only 50% (Fig. 3). Similar results were obtained during study of the dependence of ADP compartmentation in the active site of mt-CK on ADP concentration [147]. These data indicate lack of direct adenine nucleotide transfer within the active sites of these proteins. These data can be explained by the existence of diffusion restraints in the environment of these proteins; reduction in ATP and ADP concentrations promotes manifestations of these diffusion restraints and causes the increase in compartmentation in the active site of mt-CK on reduction of adenine nucleotide concentration in the medium.

Treatment of mitochondria with digitonin, which disrupts the outer mitochondrial membrane, abolished the compartmentation observed at low ATP concentrations [148]. Since these experiments were carried out in medium with low ionic strength for very short time intervals (5-10 sec), the removal of compartmentation could not be explained by solubilization of mt-CK from mitochondrial membranes or dissociation of octamers to dimers. It was concluded that direct exchange between mt-CK and ANT is absent, and compartmentation can be explained by the outer mitochondrial membrane as a permeability barrier for adenine nucleotides [148]. Other isotope experiments [83, 136, 146] also suggest lack of an adenine nucleotide compartment restricted by an mt-CK–ANT complex.

The same gradients of ADP concentration between the intermembrane space and the external medium were achieved during functioning not only of mt-CK but of mitochondrial adenylate kinase as well [78-80, 149]. Adenylate kinase is a soluble protein of the intermembrane space that is not bound to mitochondrial membranes [127]. These data indicate that mt-CK and adenylate kinase are located in a single compartment separated by mitochondrial membranes, and there are no additional diffusion barriers between the two enzymes inside this compartment. Another difficulty for direct nucleotide exchange is the fact that mt-CK and ANT use magnesium complexes of adenine nucleotides and free nucleotides as substrates, respectively [150].

Since there was no information concerning time-dependent changes in creatine kinase reactant concentrations, it was hard to analyze results of thermodynamic experiments [120-122]. So, we recently performed exper-

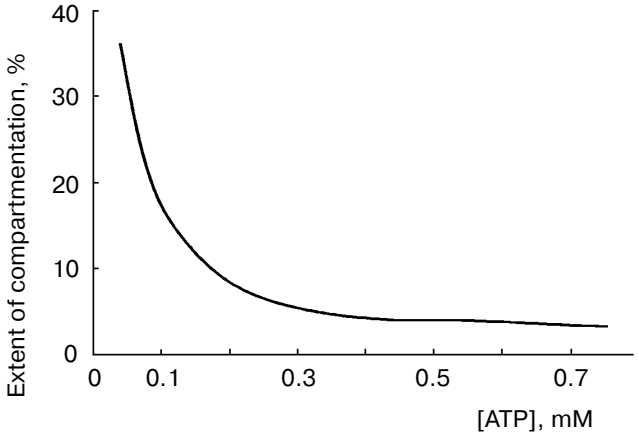

Fig. 3. Effect of ATP concentration in the incubation medium on the contribution of mitochondrial ATP to CP synthesis in heart mitochondria (adapted from [82]).

iments where such changes were monitored (Lipskaya et al., in preparation). The experimental conditions were similar to those described by Sobol et al. [122]. Our data demonstrate that exceeding of the acting mass ratio of the creatine kinase reaction reactants over K_{app} is achieved due to an increase in ADP concentration rather than an increase in CP concentration (as the structural–functional coupling hypothesis would suggest); an increase in ADP concentration was detected when the increase in CP concentration ceased. This increase was equally observed in the presence and in the absence of Cr and was attributed to mitochondrial ATPase activity increasing over the incubation time.

Indirect data suggest that in the above-mentioned reports [120-122] the exceeding of the acting mass ratio of the creatine kinase reaction reactants over K_{app} could be also achieved due to ATPase activity. For example, the authors used mitoplasts with significantly higher ATPase activity than is present in intact mitochondria (see Fig. 2 of [121]). In these experiments prolonged incubations were used, 20-60 [122] and 120-160 min [120]. Prolonged incubation of mitochondria and especially mitoplasts at 30-37°C is known to be accompanied by their swelling and uncoupling of oxidative phosphorylation followed by stimulation of ATPase activity. The ATPase reaction is irreversible; it reduces ATP concentration and increases ADP concentration. This results in the exceeding of acting mass ratio of the creatine kinase reaction reactants over K_{app}; however, the mechanism of this phenomenon is not related to functional coupling between mt-CK and ANT.

Also, the oxidative phosphorylation inhibitors used in control experiments [121, 122], oligomycin and carboxyatractyloside, are inhibitors of mitochondrial ATPase. So, it is not surprising that in their presence the acting mass ratio of reactants of the creatine kinase reaction returned to the K_{app} value [121, 122]. Other control treatments employed, anaerobiosis [122] and cyanide addition [120], inhibit (although indirectly [151]) not only respiration but also mitochondrial ATPase.

Thus, analysis of kinetic and thermodynamic arguments supporting the Saks–Jacobus hypothesis shows that they cannot be interpreted unequivocally. At the same time, direct radioisotope studies of ATP and ADP compartmentation at the active site of mt-CK [82, 136, 146-148] and some other data [78-80, 149] argue against structural–functional coupling between mt-CK and ANT.

As to structural data supporting the Wallimann–Brdiczka hypothesis, it should be noted that the existence of protein complex formation does not prove the existence of direct metabolite transfer or even metabolite compartmentation within this complex. Moreover, the interaction of the enzymes within such a complex might induce changes in tertiary and quaternary structure accompanied by changes in kinetic parameters; this might be misinterpreted as an indication for direct exchange of substrates that does not actually exist. Attempts to cross-link these postulated protein complexes during their formation were not successful [105].

It has been shown that porin and ANT are randomly distributed over the surface of the outer and inner mitochondrial membranes [113, 128]. Histochemical and immunological electron microscopy has revealed that mt-CK is located both in boundary membrane contact sites [111, 129] and on crista membranes [111, 152]. Lipskaya et al. [153] cross-linked mt-CK with mitochondrial membranes using glutaraldehyde and found two types of mitochondrial sites between which mt-CK is equally distributed (1 : 1). So, it is possible that only 50% of mt-CK molecules are localized at the contact sites. Radioisotope studies on the determination of ATP and ADP compartmentation in the active site of mt-CK also revealed maximal compartmentation of about 50% [82, 147]. These data are also consistent with the existence of two sites of mt-CK localization (in 1 : 1 ratio). The latest data also suggest that not more than 50% of mt-CK molecules are located near ANT. It is possible that this is mt-CK localized only at contact sites or only on crista membranes.

In rat heart mitochondria, the content of mt-CK octamers is 0.125 nmole per mg protein [59]. Thus, the content of ANT dimers (1.27 nmole per mg protein) [59] and porin dimers (0.35 nmole per mg protein) [78] is 10 and 3 times, respectively, higher than that of mt-CK octamers. One mt-CK octamer can kinetically serve for 40-50 ANT molecules [111]. If mt-CK were to "collect" ATP from a large number of ANT molecules during direct (even a short term) interaction [112], this would require very strong lateral diffusion of these molecules and the existence of some mechanism responsible for correct orientation of molecules during their interaction. However, the inner mitochondrial membrane largely consists of proteins [154-156] and is characterized by high viscosity, thus impeding diffusion [154]. Finally, as recently recognized, the active sites of mt-CK are located on the periphery of the enzyme molecule [157] and not exposed to the central channel cavity as proposed earlier [112].

If the number of ANT molecules (as well as number of pores in the outer mitochondrial membrane) greatly exceeds the number of mt-CK molecules and if the turnover number of each mt-CK molecule is much higher than the turnover number of ANT molecules, successful functioning of elements of this system does not require their structural interaction that would provide direct transfer of adenine nucleotides between their active sites. The location of the components of this system in a single compartment, in the intermembrane space, is the only condition needed for the effective functioning of this system. In this case, adenine nucleotides must "cruise" between active sites of mt-CK and ANT via diffusion.

Thus, at present there are no convincing data unequivocally supporting any of the considered hypotheses. At the same time, many existing data contradict them.

The localization of mt-CK at mitochondrial membrane contact sites and the ability of the octamer to interact with both boundary membranes suggest the possible involvement of the octamer in the formation of these contact sites and in the regulation of the permeability of the outer membrane for metabolites. It was shown that mt-CK dimer interacts with the outer membrane more weakly than the octamer [134, 158], and it was suggested that reversible octamer–dimer transitions may play a regulatory role in the formation of the intermembrane contacts [128]. Thus, it is possible that, in addition to its catalytic function, mt-CK (together with other proteins) plays a structure-forming role in the outer membrane–inner membrane contact sites [133]. It is also possible that mt-CK can indirectly regulate pore permeability via formation of the contact sites [115, 159].

PHYSIOLOGICAL ROLE OF mt-CK AND CK BOUND TO OTHER INTRACELLULAR STRUCTURES

So far we have analyzed studies that were looking for the optimal conditions for manifestation mt-CK activity. The postulated direct transfer of adenine nucleotides between mt-CK and ANT is used to explain effective synthesis of CP catalyzed by mt-CK. However, oxidative phosphorylation is the primary process of energy generation, and so it is important to know the role of mt-CK in promotion of effective ATP synthesis in mitochondria.

CK is located at both sides of the outer mitochondrial membrane, and so it was suggested that Cr transfer from the cytoplasm to the intermembrane space may be considered as an indirect transfer of ADP, the substrate for oxidative phosphorylation, and that the main role of mt-CK may be this indirect ADP transfer [140, 143, 160].

The asynchronous flight muscles of insects, which are characterized by a high rate of contractions and a high level of aerobic metabolism, contain an evolutionary precursor of CK, arginine kinase, which is located only in the cytoplasm, but not in mitochondria [161-163]. Wyss et al. [163] interpreted this fact considering the large number of mitochondria in the flight muscles and small diffusion distances. However, the diameter of fibrils in the insect asynchronous muscles (2 μm) [164] is roughly the same as myocardial fibrils (1-2 μm) [54], and the number of mitochondria in them (40% v/v) [164] is a bit higher than in the heart (22-37%) [41] (up to 40%, as in the case of small animals [60]). The rate of energy production (QO_2·P/O) by flight muscle mitochondria is comparable with the rate for heart mitochondria [165, 166]. The adenine nucleotide content in these muscles is within the range found for vertebrate excitatory tissues [167, 168].

However, under physiological conditions the K_{app} for the arginine kinase reaction [169] in the direction of arginine phosphate (ArgP) synthesis is 10 times higher than the K_{app} for the creatine kinase reaction [15], and the ArgP/Arg ratio is lower than the CP/Cr ratio in vertebrate muscles [168]. In resting flight muscles the arginine kinase reaction is at equilibrium [168]. All these data suggest that in resting asynchronous flight muscles the concentration of free ADP may be several-fold higher than in vertebrate muscles. During flight this concentration further increases [167]. Lack of arginine kinase in flight muscle mitochondria might be explained mainly by the presence of relatively high concentrations of free ADP in the muscle cytoplasm, under these conditions a mitochondrial isoenzyme of arginine kinase not being needed. In flight muscle the rate of oxidative phosphorylation is known to be regulated by the activity of various dehydrogenases (which are activated during transition from rest to muscle contraction) rather than by ADP concentration [170]. Special mechanisms that overcome the inhibitory effect of high ADP concentration on actomyosin ATPase also exist [171].

Perhaps the difference in the equilibrium conditions of the two reactions is the major reason for the evolutionary change from arginine kinase to CK. Creatine kinase may provide a higher ATP/ADP ratio and higher phosphoryl potential in cytoplasm than the arginine kinase reaction.

Thus, the use of CK rather than arginine kinase is very advantageous because CK can maintain a high ATP/ADP ratio in the cytoplasm. However, this raises a new problem, because ADP concentration in the cytoplasm is low and the outer mitochondrial membrane has limited permeability. The appearance of mt-CK in the intermembrane space of mitochondria was obviously the solution to this problem.

Figure 4 shows a scheme for the functioning of mt-CK that logically summarizes the considerations given above. Since the gradient generated at the outer membrane lies within the range of micromolar concentrations, the mitochondrial intermembrane space concentrations of three CK substrates, ATP, CP, and Cr, must not differ from their concentrations in the cytoplasm. In the cytoplasm of skeletal and heart muscles the creatine kinase reaction is close to equilibrium over a wide range of conditions [53, 54, 172]. This means that mt-CK cannot generate an intermembrane space concentration of the fourth substrate, ADP, which at any given moment would be higher than that in the cytoplasm. The closer the mt-CK is to the equilibrium condition, the closer is the intermembrane space ADP concentration to the concentration of ADP in the cytoplasm.

Thus, the specific function of mt-CK is the rapid equilibration of ADP concentration and, consequently, equilibration of intermembrane space ATP/ADP ratio with values that exist in the cytoplasm [173]. Since this system operates without amplification, mt-CK activity provides objective information for the oxidative phosphorylation system concerning the current value of the phos-

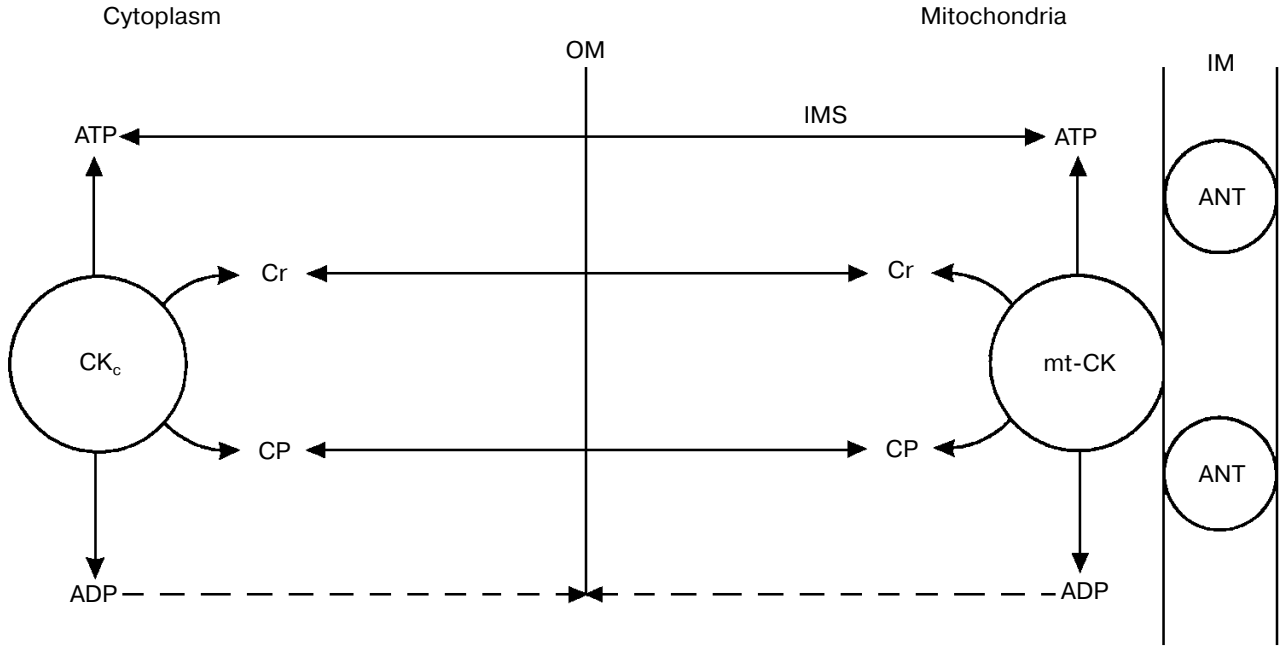

Fig. 4. Scheme illustrating the role of mt-CK in equilibrating the concentration gradient of ADP across the outer mitochondrial membrane (OM): i) direct nucleotide exchange between mt-CK and ANT is absent; ii) concentrations of ATP, Cr, and CP in the intermembrane space (IMS) are the same as in the cytoplasm (as indicated by solid bi-directional arrows crossing OM); iii) OM has limited permeability for ADP (dashed arrows which do not cross OM); iv) creatine kinase reactions in the cytoplasm (CK_c) and in mitochondria are operating near equilibrium condition (arrows indicate both directions of the reaction); v) mt-CK activity results in the equilibration of ADP concentration between IMS and cytoplasm; (IM is the inner mitochondrial membrane).

phoryl potential in the cytoplasm. In the bioenergetic model describing events that occur during a contraction–relaxation cycle in muscle, such a feedback mechanism is sufficient for maintenance of energetic balance [174, 175]. At the same time, at low ADP concentration in muscle cells the intermembrane space ADP must stimulate mitochondrial respiration to a greater extent than it would be stimulated in the presence of the same cytoplasmic ADP concentration but in the absence of mt-CK activity (K_m^{ADP} for ANT must be lower than K_m^{ADP} for mitochondria, which is influenced by the relative impermeability of the outer membrane).

Under physiological pH values the maximal activity of mt-CK is 2-3 times higher than the maximal rate of oxidative phosphorylation [83, 176]. It is possible that oligomeric forms of mt-CK differing in their kinetic constants [141] are needed to maintain the mitochondrial creatine kinase reaction close to equilibrium over a wide range of conditions.

The maximal rate of oxygen consumption by heart is about 49-56 μg-atoms O/min per g of wet weight. Under resting conditions, heart consumes about 4 μg-atoms O/min per g of wet weight [177]. This is less than 10% of the maximally possible oxygen consumption. Under medium working load, rat heart consumes 12-23 μg-atoms O/min per g of wet weight [178], this being 25-40% of the maximally possible respiration rate that is achieved only under stress conditions. It was found that under medium working load, when coronary blood flow does not limit substrate availability, a 2-3-fold increase in the respiration rate of perfused heart is not accompanied by changes in the cytoplasmic concentrations of the reactants of the creatine kinase reaction [177, 179], which is in nearly equilibrium state. It is possible that under these conditions mt-CK is also in nearly equilibrium state. *In vivo* ^{31}P-NMR experiments on transgenic mice lacking MM-CK [140, 143] support this suggestion. At the same time, maximal working loads may shift mitochondrial and cytoplasmic creatine kinase reactions off the equilibrium state [180-182], although even at submaximal working loads a difference between systolic and diastolic metabolite content is observed only during heart perfusion with glucose but not with pyruvate [183]. According to Kushmerick's calculations, deviation of the creatine kinase system from the equilibrium state in fast twitch skeletal muscles is very small and transient even under maximal working loads [175]. Any significant deviation of the creatine kinase reaction from equilibrium means a sharp reduction in its effectiveness as an energy buffer because only at equilibrium, when $\Delta G = 0$, phosphoryl

exchange between ATP and CP occurs without energy loss by the system. If free ADP content in the cytoplasm increases under maximal working loads, the difference in ADP concentrations between the cytoplasm and the intermembrane space must be relatively lower [78], and the role of mt-CK in equilibration of ADP concentration between these compartments will be less important.

It is possible that the physiological role of other CKs bound to intracellular structures in ATP consuming compartments also consists in rapid equilibration of local ADP concentrations (which appear due to functioning of corresponding ATPases) with cytoplasmic ADP concentration. From this viewpoint the appearance of local CKs is an adaptation mechanism to complex structural organization of proteins in the cell generating additional diffusion constraints inside some protein complexes; these constraints may become significant for substances that are present in low concentrations in the cell.

As in the case of mt-CK, the closer the local creatine kinase reaction is to the equilibrium state the more effective is its functioning. For skeletal muscle myofibrils the CK/myosin ratio is 1 : 40 [40], whereas in heart myofibrils this ratio may reach 1 : 10 [184]. However, in myofibrils of rat heart and chicken fast twitch muscle, CK activity is 8- and 2.3-fold higher than ATPase activity, respectively [41]. Thus, myofibrils follow the same principle as mitochondria: the number of CK molecules is much less than that of myosin molecules (the number of ANT in mitochondria), whereas CK activity is much higher.

The proposed model suggests that mt-CK, soluble cytoplasmic CK, and structure-bound MM-CK do not function independently as the hypothesis on the transport function of the creatine kinase system suggests; they are in constant interaction.

This review clearly demonstrates that in spite of more than 70 years of history, many aspects of the functioning of the creatine kinase system still require further investigations. First, there are many unanswered questions concerning mt-CK. The role of mt-CK in mitochondrial membrane contact sites remains unclear. The role of oligomeric forms, as well as the possibility of their interconversion in native mitochondria, also requires better documentation. Study of the creatine kinase reaction and other mitochondria peripheral kinase reactions may provide important information on nucleotide compartmentation in the intermembrane space and the physiological importance of this phenomenon [149].

Important information may be obtained during studies of the creatine kinase system in non-muscle tissues and cells [185] including brain, which has a different isoform spectrum of cytoplasmic and mitochondrial CKs than muscle [186, 187], and in muscle cells of various animal species representing different evolutionary stages [60]. It is important to evaluate changes in the creatine kinase system under various pathological conditions [188-191] and to compare functions of CK and its evolutionary precursor, arginine kinase [13, 186, 192]. The importance of gene engineering and NMR methods for studies of the creatine kinase system cannot be overestimated [140, 143, 193, 194].

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